

Metabolic profiling reveals ethylene mediated metabolic changes and a coordinated adaptive mechanism of ‘Jonagold’ apple to low oxygen stress

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Abstract

Apples are predominantly stored in controlled atmosphere (CA) storage to delay ripening and prolong their storage life. Profiling the dynamics of metabolic changes during ripening and controlled atmosphere storage is vital for understanding the governing molecular mechanism. In this study, the dynamics of the primary metabolism of 'Jonagold' apples during ripening in regular air (RA) storage and initiation of CA storage was profiled. 1-methylcyclopropene (1-MCP) was exploited to block ethylene receptors and to get insight into ethylene mediated metabolic changes during ripening of the fruit and in response to hypoxic stress. Metabolic changes were quantified in glycolysis, the tricarboxylic acid (TCA) cycle, the Yang cycle, and synthesis of the main amino acids branching from these metabolic pathways. Partial least square discriminant analysis of the metabolic profiles of 1-MCP treated and control apples revealed a metabolic divergence in ethylene, organic acid, sugar and amino acid metabolism. During RA storage at 18 °C, most amino acids were higher in 1-MCP treatment, whereas 1-aminocyclopropane-1-carboxylic acid (ACC) was higher in the control apples. The initial response of the fruit to CA initiation was accompanied by an increase of alanine, succinate and glutamate, but a decline in aspartate. Furthermore, alanine and succinate accumulated to higher levels in control apples than 1-MCP treated apples. The observed metabolic changes in these interlinked metabolites may indicate a coordinated adaptive strategy to maximize energy production.

Keywords: Jonagold, Metabolomics, Metabolic profiling, 1-MCP, Controlled atmosphere

31 **Abbreviations**

32 1-MCP, 1-methylcyclopropene

33 ACC, 1-aminocyclopropane-1-carboxylic acid

34 ACO, ACC oxidase

35 ACS, ACC synthase

36 CA, controlled atmosphere

37 mETC, mitochondrial electron transport chain

38 PLS-DA, partial least square discriminant analysis

39 RA, regular air

40 TCA, tricarboxylic acid

Introduction

During ripening of climacteric fruits, ethylene biosynthesis switches from its basal production rate into an autocatalytic upsurge initiating a series of biochemical, physiological, and structural changes which modify the appearance, aroma, texture and nutritional quality of the fruits (Grierson 2013). Besides ripening, ethylene regulates many aspects of plant growth, development and responses to biotic and abiotic stresses (Grierson 2012).

Low temperature storage plays a major role in reducing the metabolism of fruits and vegetables (Lurie 2002). However, there is an optimal storage temperature, depending on the type of the fruits and cultivar, below which freezing or chilling injury develops. For apples, the recommend storage temperature ranges between -0.5 °C and 4 °C (Watkins 2003). Plants adapt to low temperature by accumulating metabolites that function as osmoprotectant, free radical scavenger, and membrane stabilizer (Cook et al. 2004, Kaplan et al. 2004).

Low temperature in combination with controlled atmosphere (CA), low O₂ with slightly elevated CO₂ level, is predominantly used for long term storage of apples and pears (Beaudry 1999). CA reduces respiration and ethylene production, and preserves firmness and colour of apples (Herregods 1989, Kader 1980). Furthermore, previous studies conducted on specific segments of the metabolic pathway, demonstrated the diverse and broad effect of O₂ and CO₂ in the electron transport chain (Nanos and Kader 1993, Saquet et al. 2000), glycolysis (Kerbel et al. 1988), tricarboxylic acid (TCA) cycle (Shipway and Bramlage 1973), ethanolic fermentation (Ke et al. 1994), Yang cycle (Bulens et al. 2014) and amino acid metabolism (Hansen et al. 2001). Low O₂ storage reduces cytosolic pH and energy charge of pears (Nanos and Kader 1993) and apples (Saquet et al. 2000). In melon, hypoxia created at the centre of the fruit resulted in the accumulation of alanine, ethanol, sucrose and other organic acids such as pyruvate and fumarate (Biais et al. 2010). Accumulation of fructose 6-phosphate and depletion of fructose 1,6-bisphosphate was observed in pears stored under elevated CO₂ atmosphere at 20 °C (Kerbel et al. 1988). While higher CO₂ level reduced oxidation of α -ketoglutarate, succinate and fumarate in apple mitochondria, the same condition stimulated malate oxidation (Shipway and Bramlage 1973). Under very high CO₂ and low O₂ storage fermentative pathway is induced resulting in accumulation of acetaldehyde and ethanol (Ke et al. 1994, Saquet and Streif 2008), generating off-flavour.

1-methylcyclopropene (1-MCP), a potent ethylene inhibitor, extends the storage life of apples by suppressing ethylene biosynthesis and perception (Blankenship and Dole 2003, Kader 1995, Watkins 2006) and subsequent physiological and biochemical processes. 1-MCP treatment combined with CA storage is more effective in maintaining the firmness of the fruit than 1-MCP treatment with RA storage (Watkins et al. 2000). However, the efficacy of 1-MCP depends on several factors such as cultivar, degree of maturity, treatment temperature and storage temperature and duration (Blankenship and Dole 2003, Bulens et al. 2014, DeEll et al. 2002). Also, 1-MCP negatively influences aroma

production (Ferenczi et al. 2006), can have a positive, negative or negligible effect on soluble solid concentration (SSC) content (Watkins 2006), and increases the CO₂ injury (Fawbush et al. 2008) and flesh browning of some apple cultivars such as ‘Empire’ (Jung and Watkins 2011). 1-MCP and CA storage affect the primary metabolism which is of particular interest due to its central role in energy production and supply of carbon precursors (Defilippi et al. 2004, Fan et al. 1999).

Metabolomics involves a complete and simultaneous analysis of the metabolome of a biological tissue (Fiehn 2002) which allows to study interconnected metabolites embedded in a metabolic network. In postharvest research, metabolomics can be used to get an in-depth understanding of various postharvest processes. Metabolic profiling has already been employed to study the underlying biochemical mechanisms accompanying fruit development (Dai et al. 2013, Katz et al. 2011, Moing et al. 2011), ripening (Lombardo et al. 2011), and stress responses of plants (Ampofo-Asiama et al. 2014, Kaplan et al. 2004, Pedreschi et al. 2009, Shulaev et al. 2008). In peaches, postharvest ripening was accompanied by a decline of 2-oxoglutarate, sorbitol and most amino acids, but by stable levels of malate, citrate, fumarate and sucrose (Lombardo et al. 2011). Complete reconfiguration of primary metabolism was also observed during tomato fruit development and ripening (Carrari and Fernie 2006, Oms-Oliu et al. 2011). Similar approaches were used to probe metabolic changes involving storage disorder of apples and pears. These include development of internal browning in ‘Braeburn’ apple (Hatoum et al. 2014, Lee et al. 2012), superficial scald in ‘Granny Smith’ apple (Rudell et al. 2009), core breakdown in ‘Conference’ pear (Pedreschi et al. 2009), and recently ‘Soggy breakdown’ during low temperature storage of ‘Honeycrisp’ apple (Leisso et al. 2015).

Bulens et al. (2014), by applying different gas conditions, temperatures and 1-MCP, demonstrated the rapid dynamics of ethylene metabolism of ‘Jonagold’ apple during the initial adaptation period of CA storage. They showed that the fast increase and subsequent decline of ethylene during CA initiation was mediated by the change in enzymatic activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS). This enzyme needs Pyridoxal 5'-phosphate (PLP) as an essential cofactor (Boller et al. 1979). The available information on the precise effect of this adaptation period on the primary metabolism of apples is still very limited. This information would be useful in getting a better understanding of stress response of the fruit required to optimise storage conditions.

The objective of this work was to study the dynamics of metabolic changes during ripening of ‘Jonagold’ apple during RA storage at 18 °C and during initial periods of CA storage at 1°C. 1-MCP was exploited to block ethylene receptors and to get insight into ethylene mediated metabolic changes during ripening of the fruit and in response to hypoxic stress.

Materials and methods

Plant material

Apple fruit (*Malus x domestica* Borkh., cv. Jonagold) were harvested on October 1, 2012 from an orchard in Rotselaar, Belgium, during the commercial harvest window for long term storage of

'Jonagold' apple. The harvest period was determined by the Flanders Centre of Postharvest Technology (VCBT, Heverlee, Belgium) based on a combination of firmness, starch, sugar and acid measurements. After cooling overnight at 1 °C, the apples were treated with 1-MCP (1 $\mu\text{L L}^{-1}$) for 24 h inside 500 L air tight containers.

Experimental setup

The experimental setup was designed to study the metabolic changes during ripening in RA (18 °C) storage and during the initial adaptation period of CA storage (1°C). Two parallel experiments were conducted. In experiment one, in contrast to experiment two, the fruit metabolism was not suppressed by either temperature or CA conditions. This allowed to study the impact of 1-MCP in more detail.

Experiment 1: storage at 18 °C

In this experiment, 1-MCP treated and control apples were stored for one week under RA (20.8 % O₂, 0.03 % CO₂) at 1 °C (cold storage) followed by a four weeks RA storage at 18 °C. Samples were collected after 1, 5, 14, 21 and 28 days of storage at 18 °C.

Experiment 2: storage at 1 °C

In this part of the experiment, after two weeks of cold storage, the 1-MCP treated and control fruit were either transferred to CA storage (1 % O₂, 3 % CO₂) at 1 °C or kept as a control under RA at 1 °C. Samples were collected on 1, 8, 17, and 21 days after initiation of the CA storage conditions. During the initial cold storage period, fruit were sampled at harvest, after one week of cold storage and after two weeks, just before the fruit was moved into CA.

Storage atmospheres were established by flushing six litres air tight plastic jars each containing five replicate fruit with humidified gas mixture of the targeted composition. The gas mixtures were prepared by mixing pure O₂, CO₂ and N₂ gases using an automated gas mixing panel (Storage control system, MeBioS, Belgium). To avoid interruption of the CA atmosphere while sampling, the jars were connected in series and samples were always taken from the last jar leaving the others intact.

Gas exchange measurements

Respiration and ethylene production rates were measured on the intact apples as described in Bulens et al. (2011). Individual apples were placed inside 1.7 L airtight glass jars fitted with inlet and outlet connection and flushed for three hours to establish the required humidified gas composition. After this initial flushing period, the gas flow was stopped and the gas composition inside the jars was measured. After an additional incubation period, of a length depending on the applied temperature and gas composition at which the measurement took place, the changed gas composition was measured again. The respiration and ethylene production rates were calculated by taking the difference of the gas measurements between the two time points and normalized by the weight of the individual apples and incubation time.

Metabolite analysis

Tissue samples for metabolite measurement were taken from the outer cortex of the apples, quenched by liquid nitrogen and stored at -80 °C. The frozen samples were crushed to a fine powder using mixer mill (Retsch, MM 200) vibrating at a frequency of 30 Hz. Metabolites were extracted by adding 1 mL of cold methanol (-20 °C) to 200 mg of the prepared powder and incubating at 70 °C in the thermomixer (Eppendorf AG, Hamburg, Germany) for 15 min (1400 rpm). Next, the samples were centrifuged at 4 °C for 20 min (14 500 rpm) to separate the cell debris from the extract. 100 µL of the supernatants was transferred into 1.5 mL eppendorf tubes into which 100 µL of internal standard mixture containing phenyl-β-D-glucopyranoside (1 mg mL⁻¹ in methanol) and 3-(4-hydroxyphenyl)propionic acid (0.3 mg mL⁻¹ in methanol) was added. Finally, the aliquots were dried under a stream of N₂ (g) at 50 °C (Stuart, sample concentrator (SBH CON/1), Bibby Scientific Limited Stone, and Stanffordshire, UK).

Two step derivatization, methoxymation followed by silylation, was used (Roessner et al. 2000, Rudell et al. 2009). For methoxymation, 125 µL of methoxyamine hydrochloride (20 mg mL⁻¹ in anhydrous pyridine) was added to the dried samples. The mixture was incubated at 30 °C for 90 min (1400 rpm) in the thermomixer (Eppendorf AG, Hamburg, Germany). Subsequently, 125 µL BSTFA was added to the mixture, incubated at 37 °C for 30 min (1400 rpm) in the thermomixer (Eppendorf AG, Hamburg, Germany) and centrifuged (5417R centrifuge, Eppendorf AG, Germany) for 3 min (14 500 rpm). Finally, 100 µL of the derivatized sample was transferred into glass vials containing deactivated glass inserts.

1 µL of derivatized samples was injected into GC 7890A coupled with 5975C MS (Agilent Technologies, Palo Alto, CA, USA) using the auto sampler. The chromatographic separation was performed on a 30 m HP-5ms column (Supelco, Bellefonte, CA, USA) of 0.25 mm internal diameter and 0.25 µm film thickness. The samples were volatilized at the injection temperature of 220 °C inside the deactivated glass liner (SGE Analytical Science, Australia). Helium was used as a carrier gas with constant flow of 1 mL min⁻¹. Pulsed splitless injection was applied for less abundant metabolites while a split ratio of 500:1 was used for most abundant ones. In splitless mode, the oven temperature was held initially at 50 °C for 1 min, ramped at 10 °C min⁻¹ to 310 °C and held for 13 min at 310 °C. In split mode, the oven temperature was kept at 120 °C for 1 min and ramped to 310 °C by 10 °C min⁻¹ and held at this temperature for a further 6 min. The mass selective detector (MSD) was operated in the electron ionization (EI) mode with quadrupole and MS ion source temperatures maintained at 150 and 230 °C respectively. The mass spectra ranging from m/z 30 to m/z 600 were recorded.

Quality control samples were included with each injection sequence containing 20 samples. These include a solvent blank containing hexane, a method blank which contains all the solvents used for analysis, a reference standard mixture, and a Grob mixture (Barry 2004). The inlet liner and septum were monitored daily for contamination and changed every 40 injections.

Compound identification was performed by comparing the retention indexes and the deconvoluted mass spectra (AMDIS, National Institute of Standards) of the compounds against an in-house built mass spectral library and commercial Fiehn (Kind et al. 2009) and NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) libraries. The metabolites were quantified based on peak areas of their respective characteristic ions using MSD ChemStation software (Agilent Technologies, Palo Alto, CA, USA). To normalise for small variations due to derivatization and GC-MS analysis, phenyl- β -D-glucopyranoside and 3-(4-hydroxyphenyl)propionic acid were used as internal standards for high and less abundant metabolites, respectively. In addition to the internal standards, peak areas were also normalized by the sample's fresh weight.

Multivariate analysis

The relative responses of the metabolites were mean centred and standardized to unit variance before using them for multivariate statistical analysis. To reveal the structure in the multivariate dataset, partial least square discriminant analysis (PLS-DA) was conducted using The Unscrambler® X software (version 10.3, CAMO A/S, Trondheim, Norway). PLS is a multivariate regression technique which tries to do two things at a time. On one hand it reduces the dimensionality of a complex data set of highly correlated variables thus summarising the most important information into just a few dimensions. As such it is used for visualisation and provides insight in the complex structure of the data. At the same time it is a regression technique which looks for a relationship between the multivariate data and certain response variables using the reduced variable space. Metabolites were used as predictor variables while the categorical variables representing the treatment groups and the continuous variable time were used as response variables. Metabolites to be shown in the PLS-DA biplot are selected based on stability of the model coefficients of important metabolites ($p < 0.05$) determined by jack-knife based resampling, and are used to discriminate between groups (Martens and Martens, 2000). Paired t-test (JMP® (Version 11, SAS Institute Inc., Cary, NC) was used to identify metabolites that have significant difference between the 1-MCP treated and control apple. The heat map visualization was made by using VANTED software (Junker et al. 2006).

Results

1-MCP inhibits respiration and ethylene biosynthesis

To monitor the dynamics in the physiology status of the fruit, respiration and ethylene production rates were measured. As expected, 1-MCP effectively reduced the respiration and ethylene production rate of 'Jonagold' apples during RA and CA storage (Fig. 1). This result is in agreement with previous reports (Bulens et al. 2012, Fan et al. 1999, Mattheis et al. 2005). Ethylene production drastically increased in control apples reaching a peak value of $1.56 \text{ nmol kg}^{-1} \text{ s}^{-1}$ in the second week of RA at 18°C (Fig. 1A). Although ethylene production of 1-MCP treated apples tended to increase after two weeks of RA at 18°C , its production rate was still tenfold lower than in control apples. During cold

storage (Fig. 1C), 1-MCP effectively arrested the sharp increase of ethylene production in control apples, which was already considerably lower than ethylene production at 18 °C. This production rate was further suppressed by CA storage.

Temperature had a major effect on the respiration rate of the fruit. After transferring the control apples from 1 °C to 18 °C, the respiration rate increased drastically (Fig. 1B). The respiration rate further increased during the first week at 18 °C and declined afterwards. While 1-MCP substantially reduced respiration rate during the first three weeks at 18 °C, following a similar pattern as ethylene, respiration rate tended to rise again after three weeks of storage. CA storage (Fig. 1D) further lowered the respiration rate of both 1-MCP treated and control apples showing the additional benefits of applying CA in reducing respiration rate of apples.

Metabolic analysis revealed metabolic changes distinct to 1-MCP treatment during RA storage at 18 °C

By using GC-MS based targeted metabolic profiling, 32 polar metabolites from different metabolic classes including organic acids, amino acids, sugars, sugar alcohols and polyphenols were detected and quantified (Table 1).

At first, a general view using PLS-DA analysis (Fig. 2) of the metabolic profiles of 1-MCP treated and control apples revealed a metabolic divergence in ethylene, organic acid, sugar and amino acid metabolism specific to each treatment. Moreover, the metabolic profiles of the two groups diverged with increasing storage time. The scores from the PLS-DA analysis (Fig. 2, closed symbols) showed that 1-MCP treated and control apples were clearly separated by their metabolic profiles throughout the four weeks of RA storage at 18 °C. The correlation loadings (Fig. 2, open symbols) revealed a close positive association between most amino acids and 1-MCP treatment. In contrast, 1-aminocyclopropane-1-carboxylic acid (ACC) was positively correlated with the control apples. Citramalate and ribose increased significantly with time, particularly in the control fruit, while succinate was negatively correlated to storage time.

1-MCP differentially affects organic acids and sugars metabolism during RA storage at 18 °C

Turning now to a more detailed analysis, Fig. 3 shows that 1-MCP treatment diversely affected the metabolism of organic acids and sugars. Irrespective of the storage temperatures, the accumulation of ACC was completely suppressed in 1-MCP treated apples (Fig. 3A). However, during the first week of cold storage, ACC sharply increased in the control fruit signifying the conversion of ACC to ethylene to be slower than its formation from S-adenosyl-L-methionine (SAM). The control fruit exhibited higher ACC level at 1 °C storage as compared to 18 °C. Phosphate and maleate were lower in the control apples (Figs. 3B & D). Citramalate sharply increased with storage time particularly in the control fruit (Fig. 3C).

Among the TCA cycle intermediates, 1-MCP effectively retained the levels of malate, citrate and fumarate, but not succinate (Figs. 3E-3H). Unlike citrate and malate, succinate significantly decreased

with storage time in both groups (Fig. 3G). By contrast, no significant difference was observed in pyruvate, quinate, shikimate and chlorogenate between the two groups (Figs. 3I-3L).

Glucose substantially accumulated in 1-MCP treated apples (Fig. 3M) during RA storage at 18 °C, as compared to the control apples. Fructose was higher in the 1-MCP treated apples (Fig. 3N), although it had not accumulated as the same level as glucose. The sucrose content of the control apples decreased during the first week of cold storage (Fig. 3O), but restored close to its level at harvest during the first week of RA storage at 18 °C. On the other hand, the sucrose content of 1-MCP treated apples started to decline immediately after transferring to 18 °C. Interestingly, the control apples managed to maintain a higher sucrose content throughout the four weeks of RA storage as compared to the 1-MCP treated fruit. The decrease in sucrose level after two weeks of storage suggested the metabolic balance of sucrose metabolism inclined towards sucrose degradation. Ribose increased substantially at 18 °C storage, particularly in control apples (Fig. 3P).

Amino acids distinctively accumulate in 1-MCP treated apples during RA storage at 18 °C

1-MCP treated apples were characterized by a high content of most amino acids (Fig. 4) as compared to the control fruit. Generally, the amino acid content of the control apples declined during RA storage at 18 °C, whereas glutamate was the only amino acid which steadily declined in both groups (Fig. 4C). Isoleucine peculiarly accumulated in both groups, but to a higher extent in 1-MCP treated apples as compared to control fruit (Fig. 4E). The accumulation of isoleucine in the control apples is in agreement with previous reports (Defilippi et al. 2005, Magné et al. 1997, Sugimoto et al. 2011). Valine accumulated significantly in the 1-MCP treated but not in the control apples (Fig. 4F). On the other hand, most amino acids in 1-MCP treated fruit, started to decline after three weeks at 18 °C (Fig. 4). This coincides with increased ethylene production (Fig. 1A).

There was no significant difference in erythritol among the two groups (Fig. 4L). The control fruit had higher xylitol during the initial two weeks but reached similar level as the 1-MCP treated fruit afterwards (Fig. 4N). 1-MCP treated apples exhibited higher content of inositol and sorbitol after two and three weeks of RA storage at 18 °C respectively (Figs. 4M & 4O).

Metabolic profiling revealed distinct responses to RA and CA storage at 1°C

As compared to RA storage at 18 °C, the metabolic differences between the control and 1-MCP treated apples stored in RA and CA storage at 1°C diverge to a lesser extent, though the differences were still sufficient to discriminate the two groups.

The PLS-DA biplot (Fig. 5) depicts a clear separation, based on their metabolic profiles, between 1-MCP treated and control apples stored under RA and CA storage for a period of three weeks. Alanine, isoleucine and succinate were positively associated with CA storage. 1-MCP treated apples stored under CA storage were positively associated with glutamate, valine, serine, threonine and asparagine suggesting the additional role of CA in maintaining the level of these amino acids. Similar effect of 1-MCP on amino acids content has been reported for CA stored ‘Empire’ apples (Lee et al. 2012). On

the other hand, pyruvate was positively correlated with control apples stored under RA while ACC was positively correlated with control apples irrespective of the storage conditions. Fig. 6 shows the four metabolites which changed considerably in response to induction of CA. On initiation of CA, alanine, succinate and glutamate immediately increased in control apples (Fig. 6). However, aspartate rapidly declined when the CA condition was imposed. Interestingly, the alanine content of the control apples was higher than that of 1-MCP treated apples.

Metabolic profiles during RA storage at 1 °C

Fig. 7 shows the heat map representation of the metabolic profiles of 1-MCP treated and control apples during five weeks of RA storage at 1 °C. Some clear differences were noted between the control and 1-MCP treated apples during RA at 1°C, although for most metabolites the profiles were less distinct as compared to RA storage at 18 °C. ACC significantly accumulated in the control apples. Similar to RA storage at 18 °C, aspartate, isoleucine, glutamate, glycine, serine, threonine and valine were higher in 1-MCP treated apple. However, there was no significant difference in alanine, asparagine, β -alanine and γ -aminobutyric acid (GABA). Among the organic acids, malate was slightly higher in 1-MCP treated apples. Sucrose decreased during cold storage, while fructose and glucose increased.

Discussion

1-MCP arrests respiration and ethylene metabolism

The increased respiration and ethylene production of control apples during RA storage at 18 °C requires a high flux in the mitochondrial electron transport chain (mETC) and Yang cycle, respectively. As the metabolic flux through these pathways is closely coupled with the activity of the TCA cycle and the glycolytic pathway (Plaxton 1996), the induction of respiratory burst in the control apples is presumably associated with the biosynthesis or increased activity level of respiratory enzymes. A recent study showed an increased abundance of NADP-dependent malic enzyme during postharvest ripening of apples and a decline in triosephosphate isomerase and 6-phosphogluconolactonase after the climacteric peak (Shi et al. 2014). The suppressed respiration rate of both the 1-MCP treated and cold stored apples suggested reduced enzymatic activity of these enzymes by low temperature (Kruse et al. 2011) and 1-MCP. The recovery of ethylene production in 1-MCP treated apples during the later periods of RA storage at 18 °C might be due to the formation of new receptors and/or release of 1-MCP molecules previously bound to the receptors (Blankenship and Dole 2003).

1-MCP suppresses the accumulation of ACC

Lower concentration of ACC (Fig. 3A) in 1-MCP treated fruit as compared to the control fruit at both storage temperatures is probably due to the suppressed activity of ACC synthase (ACS) during cold storage (Lelièvre et al. 1997, Tian et al. 2002) and shelf life (Bulens et al. 2014). Tian et al. (2002)

showed that while low temperature stimulated the gene expression of both ACS and ACC oxidase (ACO), it increased only the enzymatic activity of the former gene transcript. The accumulation of ACC observed in this study during cold storage of control apples suggested ACO as the rate limiting enzyme at 1°C. Our result showed that, although the ACC level of the control apples declined as the fruit was transferred to 18 °C, the level was still higher than the 1-MCP treated fruit. This could be accounted for by a temperature mediated increase in ACO activity and the induction of new ACO proteins during shelf life at 18 °C (Bulens et al. 2014). In summary, the control fruit exhibited higher ACC level at cold temperature (1 °C) as compared to room temperature (18 °C). In addition, as demonstrated by high ethylene production, the flux of carbon through ethylene biosynthesis pathway was higher at the latter temperature. This result demonstrated that, under these conditions, there was no direct relationship between the actual ACC level of the tissue and ethylene production rate.

Ethylene regulates sugar metabolism during RA storage at 18 °C

Numerous researches showed the simultaneous synthesis and degradation cycle of sucrose metabolism during postharvest storage of fruits (Castrillo et al. 1992, Duque et al. 1999, Lombardo et al. 2011, Zhu et al. 2013). In this cycle, sucrose is synthesized from sucrose-6-phosphate by sucrose-phosphate phosphatase or from fructose and uridine diphosphate glucose (UDP-glucose) by sucrose synthase (SuSy), although during postharvest storage SuSy favourably proceeds in the direction of sucrose breakdown. Sucrose-phosphate synthase (SPS) catalyse the formation of sucrose-6-phosphate from UDP-glucose and fructose-6-phosphate. Sucrose is catabolized either into glucose and fructose by invertase or into UDP-glucose and fructose by SuSy.

One of the characteristic metabolic difference between 1-MCP treated and control apple was the low sucrose content (Fig. 3O) of the 1-MCP treated apple during RA storage at 18 °C. This finding argues for ethylene mediated biosynthesis of sucrose from hexose sugars. Defilippi et al. (2004) similarly found a lower content of sucrose in 1-MCP treated Greensleeves apples. Indeed, increased gene transcript and enzymatic activity of SPS by exogenously applied ethylene in preclimacteric banana (Choudhury et al. 2008) and increased gene transcript of SPS during postharvest ripening of peaches (Lombardo et al. 2011) demonstrated the link between ethylene and sucrose metabolism. Furthermore, Duque et al. (1999) showed that SPS increased until the climacteric peak of apples and declined afterwards while SuSy continued to increase during post-climacteric period. The decline of sucrose in 1-MCP treated apple following the transfer of the fruit in to 18 °C is probably due to temperature mediated activation of sucrose degrading enzymes and suppression of SPS by 1-MCP treatment. In contrast to the treated apples, the breakdown of sucrose did not result in accumulation of glucose in control fruit, owing to its utilization to sustain a high respiration rate observed at these time points.

1-MCP reduced the depletion of TCA cycle intermediates and ripening related utilization of amino acids

The activity of the TCA cycle closely reflects the physiological context in which the cycle is working (Sweetlove et al. 2010). Malate depleted in the control apple to sustain high respiration rate during RA

storage at 18 °C (Fig. 3E). During ripening malate is decarboxylated producing pyruvate in reactions catalysed by a NAD or NADP dependent malic enzyme, allowing malate and other members of the TCA cycle to be used as respiratory fuel. It has been shown that the activity and abundance of NADP dependent malic enzyme significantly accumulates during ripening of apples (Hulme et al. 1963, Shi et al. 2014). Moreover, NADPH generated by NADP-ME could be used for the biosynthesis of fatty acids, nucleotides and antioxidant defence required for ripening. Citramalate significantly increased with time (Fig. 3C). The citramalate biosynthesis pathway is not well elucidated in plants; however, in bacteria it is known to be formed from pyruvate and metabolized into isoleucine (Xu et al. 2004).

The high content of amino acids in 1-MCP treated apples as compared to the control fruit might be related to their reduced utilization by ripening related enzymes such as those involved in aroma and ethylene biosynthesis pathways (Fig. 4). It has been shown that 1-MCP could delay the onset of aroma production by up to 25 days (Ferenczi et al. 2006). Branched chain (BC) amino acids (isoleucine and valine) are important precursors for biosynthesis of BC α -keto acids which are direct precursors for formation of BC esters (Rowan et al. 1996). Aspartate, derived from the TCA cycle, is the starting point for biosynthesis of asparagine, threonine and isoleucine (Azevedo et al. 2006), and is linked to the ethylene metabolism through methionine. Therefore, the decline of aspartate in the control apples (Fig. 4A), but not in 1-MCP treated apples, suggested its utilization for biosynthesis of ripening related enzymes through these amino acids.

Glutamate has diverse physiological roles as energy source, in the biosynthesis of other amino acids, and as a signalling molecule (Forde and Lea 2007). It enters into the TCA cycle either through 2-oxoglutarate (2OG) or through the GABA shunt. The α -amino group of glutamate could be transferred to various 2-oxo acids to form other amino acids. The depletion of glutamate even in 1-MCP treated fruit (Fig. 4C) suggested the metabolism of glutamate is regulated at least partly in an ethylene independent manner. The organic acid metabolism in the TCA cycle is linked to the amino acids metabolism through the by-pass involving glutamate and GABA. The control apples had a lower content of GABA (Fig. 4J) as compared to 1-MCP treated apples. The incorporation of GABA into TCA cycle intermediates has been demonstrated using ^{14}C tracer analysis (Yin et al. 2010).

Isoleucine is a known precursor for the production of diverse aroma compounds including 2-methylbutanol, 2-methylbutanoate, 2-methylbutyl acetate and 2-methylbutanoic acid (Matich and Rowan 2007, Rowan et al. 1996). The isoleucine derived aroma compounds are major aroma components in ripening 'Jonagold' apple (Róth et al. 2007), and the incorporation of isoleucine into these aroma compounds was demonstrated using deuterium-labelled isoleucine (Rowan et al. 1996). The accumulation of isoleucine in the control fruit (Fig. 4E) is most likely due to a lower aroma biosynthesis from it relative to its production. Based on gene expression profiles of transgenic apple, Schaffer et al. (2007) reasoned that the aroma production of apples is not coordinately regulated, but instead mainly regulated at the last steps in these pathways. The higher accumulation of isoleucine in 1-MCP treated fruit and its concomitant decline following the increase in ethylene, suggested a key

role of ethylene in regulating aroma biosynthesis either at the level of isoleucine or further downstream. Studies conducted on transgenic Greensleeves apples suppressed in ethylene biosynthesis (Defilippi et al. 2005) and 1-MCP treated apples (Zhu et al. 2008), demonstrated ethylene regulates apple volatile formation by acting on alcohol acyl transferase (AAT). However, as demonstrated by the capacity of 1-MCP treated apple tissue to metabolize exogenous applied precursors, 1-MCP treatment might not completely arrest AAT activity (Ferenczi et al. 2006), and the availability of substrate and specificity towards AAT (Holland et al. 2005) might determine the final aroma composition.

Metabolic profiling revealed adaptive metabolic changes to low O₂ stress

The increase of alanine, succinate and glutamate together with a decline of aspartate was the main immediate response of the apples to CA storage atmosphere at 1°C (Fig. 6). Alanine and succinate ubiquitously accumulate in plants exposed to anoxia (van Dongen et al. 2011, Miyashita and Good 2008). Rocha et al. (2010) proposed a metabolic model to explain the simultaneous accumulation of alanine and succinate in hypoxia induced by waterlogging of *Lotus japonicas*. The initial response of ‘Jonagold’ apples to low O₂ stress, unlike *Lotus*, did not result in the accumulation of GABA. The coordinated adaptive change of alanine, succinate, aspartate and glutamate (Fig. 8) is discussed below in context of cellular energy production under limited O₂ supply.

Alanine might be produced from pyruvate by a transamination reaction either coupled to the conversion of glutamate into 2OG or to the conversion of GABA into succinic semialdehyde. Since the latter takes place through the less energy efficient GABA shunt, the alanine aminotransferase (AlaAT) mediated accumulation of alanine, the reaction coupled to the conversion of glutamate into 2OG, might be a more likely candidate under the given hypoxic conditions. Pyruvate activates the alternative oxidase pathway (Oliver et al. 2008) which transfers electrons without pumping protons across the inner membrane of mitochondria and hence produces less ATP than the cytochrome c oxidase pathway (Rasmusson et al. 2008). The stable level of pyruvate observed in this study under CA storage might be related to tight regulation of pyruvate under hypoxia.

The higher glutamate levels of CA stored apples could be explained together with the rapid decline of aspartate at the onset of CA storage. Aspartate could be converted into oxaloacetate (OAA) in a transamination reaction catalysed by aspartate aminotransferase (AspAT) involving the conversion of 2OG into glutamate. Streeter and Thompson (1972) showed the addition of unlabelled 2OG into radish leaves accelerated the labelling of ¹⁴C alanine concomitant with loss of aspartate, emphasizing the involvement of 2OG in the accumulation of alanine. Furthermore, the decline of alanine observed in control apples after one week of CA storage with a concomitant depletion of aspartate, underlined the potential connection between accumulation of alanine and depletion of aspartate. The accumulation of glutamate indicated that, from the two transamination reaction around glutamate, the transamination reactions catalysed by AspAT dominated over AlaAT. The higher glutamate content in

1-MCP treated apples during CA storage, as compared to the control apples, could be due to a reduced consumption for other biosynthesis processes.

Under hypoxic conditions, OAA can be converted into malate through a reversed TCA cycle, keeping the TCA cycle running by producing NAD^+ which is consumed in a reaction driving 2OG into succinyl-CoA leading to succinate (Rocha et al. 2010). The generation of NAD^+ has particular importance during CA storage as the NAD^+ regenerating capacity of the cells in the mETC will be hampered (Saquet et al. 2000). Malate could be metabolized into succinate, by entering back to the TCA cycle through pyruvate, leading to a high level of succinate observed in CA stored apples as compared to RA stored apples. However, as succinate could also be formed following the normal TCA cycle, a metabolic flux analysis study is required to quantify the magnitude of the fluxes in each direction of the pathway. Taken together, the metabolic patterns observed under CA initiation pointed towards a cellular strategy geared to maximize the energy production under hypoxic condition which constrained ATP production and NAD^+ regeneration.

Upon initiation of CA, the control apples accumulated higher alanine content as compared to the other group, suggesting a possible involvement of ethylene in regulating stress response during hypoxia. Similarly, succinate accumulated to lesser extent in the 1-MCP treated apples. It has been shown that the overexpression of ethylene response factor-type transcription factor (RAP2.2), in *Arabidopsis thaliana*, increased plant survival under anoxia (Hinz et al. 2010) by induction of genes in sugar and fermentative metabolism.

In conclusion, a targeted metabolomics approach was successfully employed to get useful insights into system wide metabolic changes during RA storage and initiation of CA of 'Jonagold' apples. Metabolic changes were evident as early as from the first day of RA storage at 18 °C and of CA initiation, which may have long-lasting consequences on the quality of the fruit. By exploiting 1-MCP as a means to impede ethylene action, insights were obtained about the metabolic processes regulated by ethylene. 1-MCP diversely affected sugars and organic acids metabolism, delayed the consumption of some TCA cycle intermediates, and leads to the accumulation of most amino acids. The onset of CA storage was accompanied by specific metabolic changes in the TCA cycle and amino acids linked to it, in response to reduced energy production due to low O_2 availability.

Authors' contributions

Conceived and designed the experiments: EB, MH, BN and AG. Carried out the experimental work, data analysis, and interpretation of results: EB and WB. Wrote the manuscript with contributions from all the authors: EB. Guided the experimental work, interpretation of the results and writing of the manuscript: MH, BN and AG. All authors read and approved the final manuscript.

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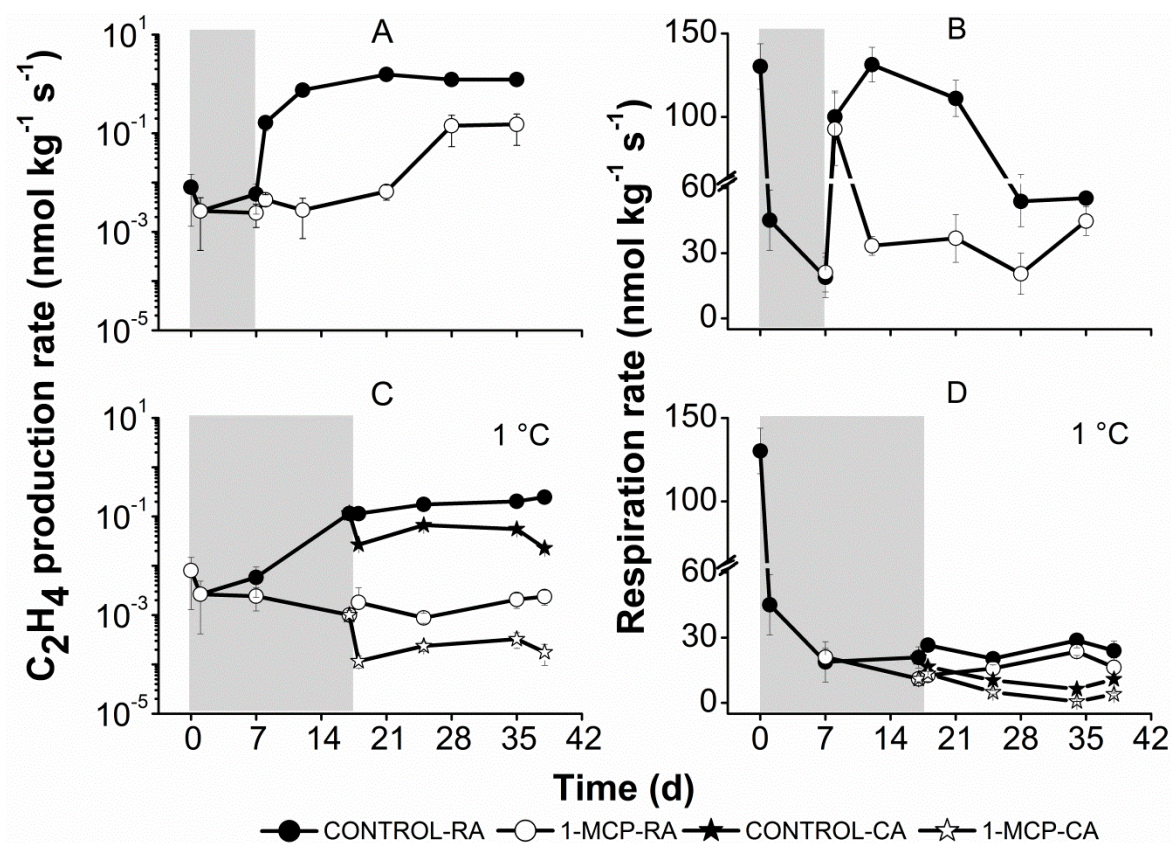


Fig. 1. Figures (A & B) represent the ethylene production and respiration rate respectively of 'Jonagold' apple during RA storage at 18 °C after one week of RA storage at 1 °C (Experiment 1). Figures (C & D) represent the ethylene production and respiration rate respectively during RA storage at 1 °C and CA storage (1 % O₂, 3 % CO₂) after 17 days of storage at 1 °C (Experiment 2). The shaded areas indicate the RA storage at 1 °C. The error bars represent standard error of the mean (n=5).

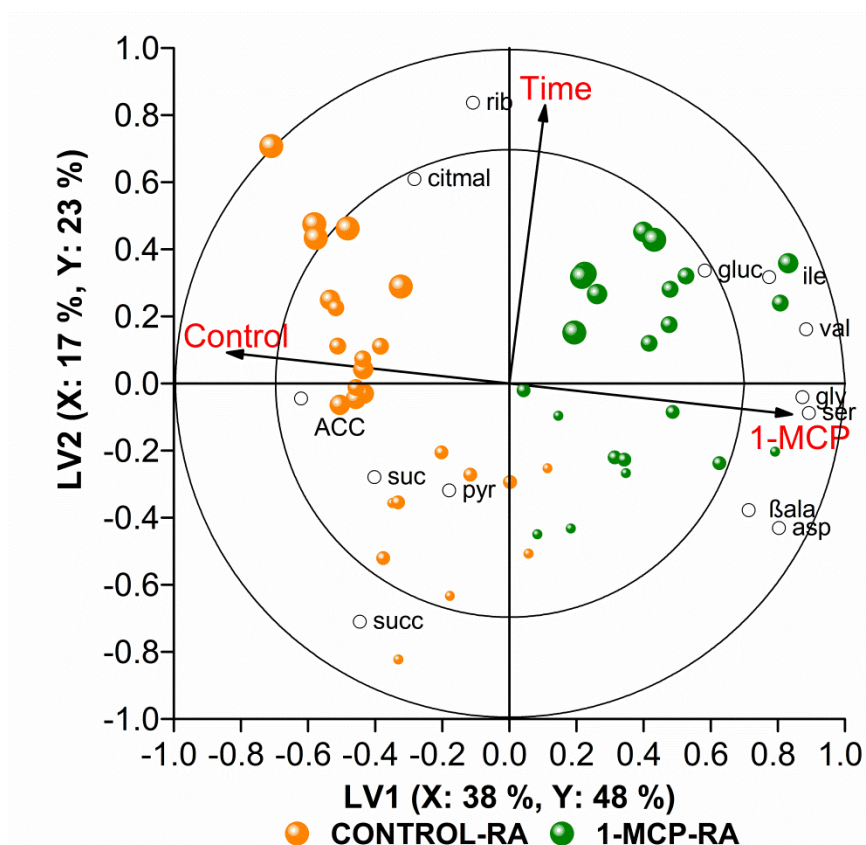


Fig. 2. The PLS-DA biplot showing a clear separation between 1-MCP treated and control ‘Jonagold’ apples based on their metabolic profiles during RA storage at 18 °C (Experiment 1). The scores (closed symbols), the X-loadings (open symbols) and Y-loadings (arrows) are superimposed. The open symbols represent the important metabolites discriminating the two groups. The size of the spheres reflects the length of the storage period. The percentage of explained variances are shown on the axes. Abbreviations: LV, latent variable and the abbreviation for the metabolites is as given in Table 1.

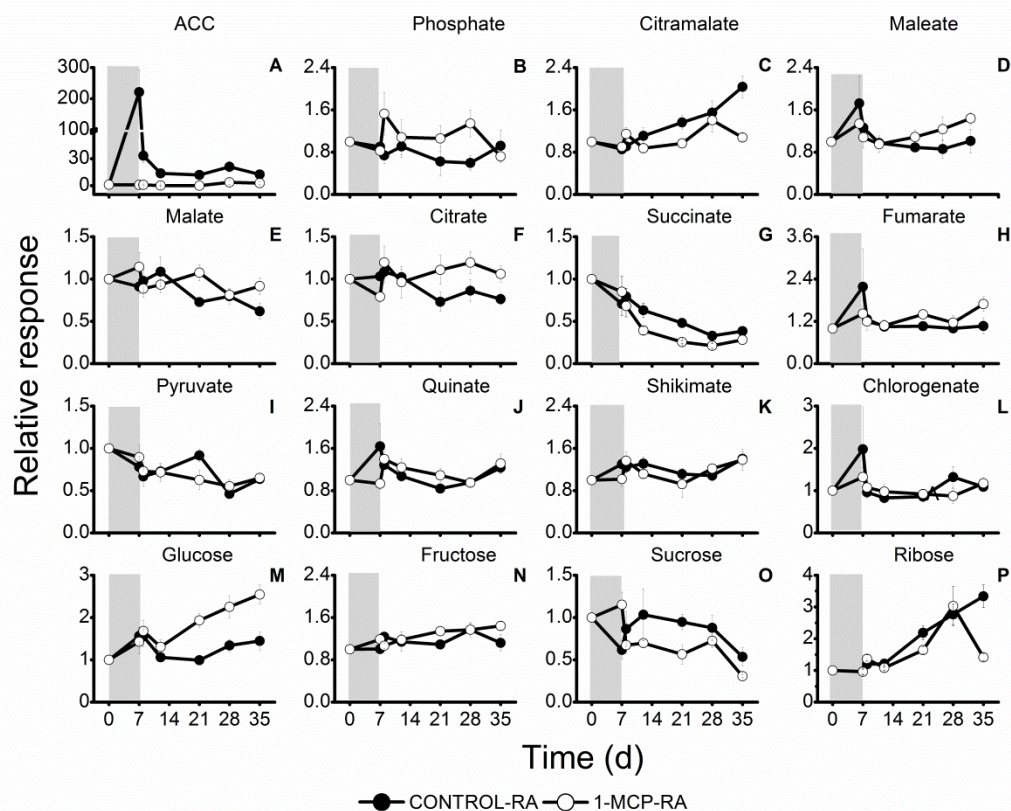


Fig. 3. The relative response of organic acids and sugars extracted from 'Jonagold' apple cortex during RA storage at 18 °C following one week of RA storage at 1 °C (Experiment 1). The relative responses denote the metabolites concentration normalized by their concentration at harvest. The error bars represent standard error of the mean (n=5). The shaded areas indicate the RA storage at 1°C.

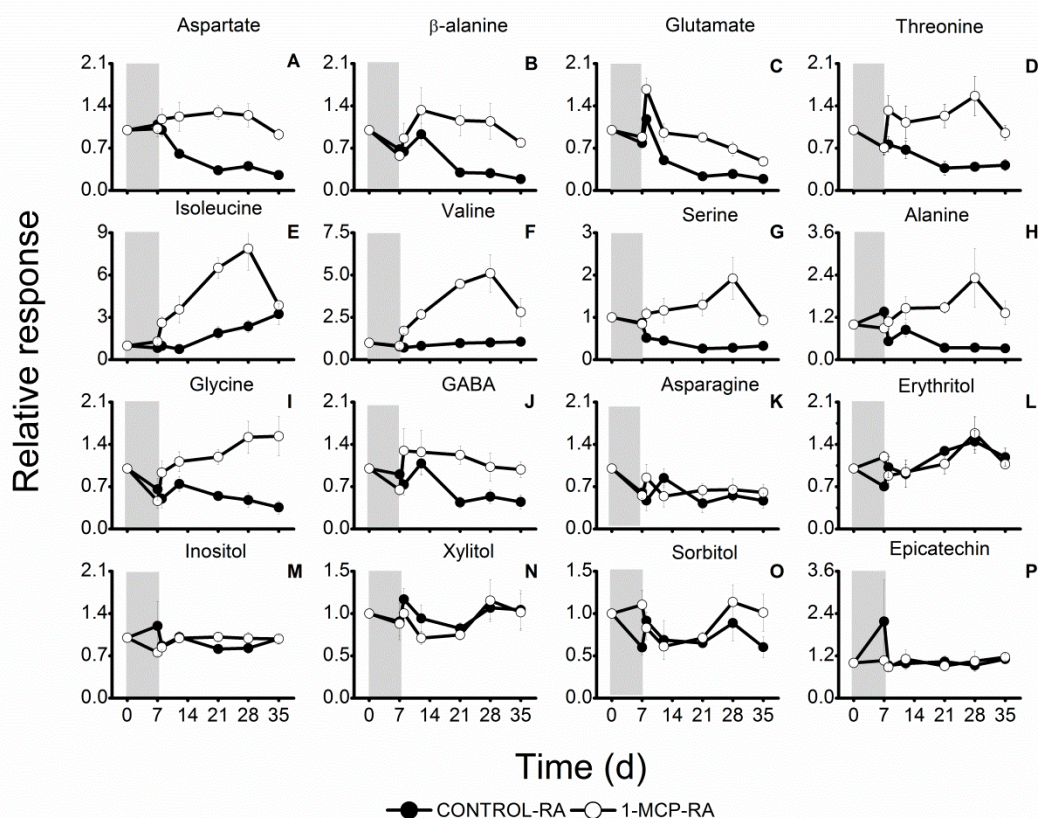


Fig. 4. The relative response of amino acids, sugar alcohols and epicatechin extracted from 'Jonagold' apple cortex during RA storage at 18 °C following one week of RA storage at 1 °C (Experiment 1). The relative response denotes the metabolites concentration normalized by their concentration at harvest. The error bars represent standard error of the mean (n=5). The shaded areas indicate the RA storage at 1°C.

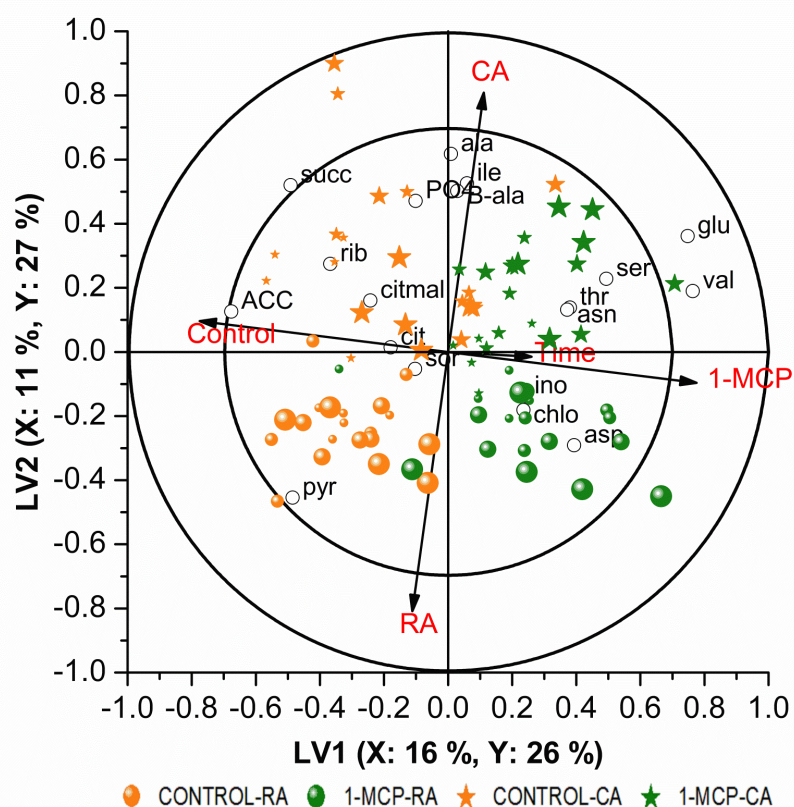


Fig. 5. 1-MCP treated and control apples stored under RA and CA storage of 'Jonagold' apple for three weeks were clearly separated based on their metabolic profiles (Experiment 2). The scores (closed symbols), the X-loadings (open symbols) and Y-loadings (arrows) are superimposed. The open circles represent the important metabolites discriminating the two groups. The size of the spheres reflects the length of the storage period. The percentage of explained variances is shown on the axes.

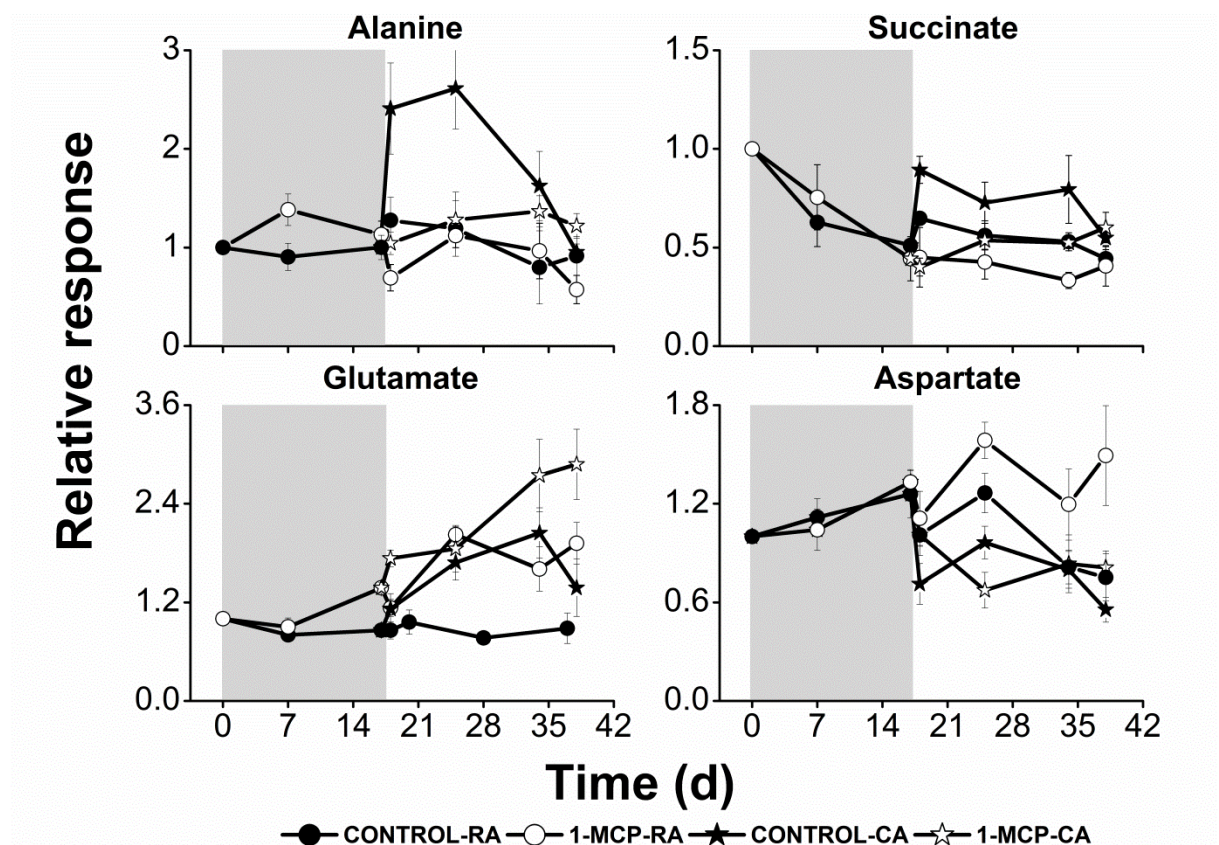


Fig. 6. The relative response of alanine, succinate, glutamate and aspartate under RA and CA storage (1 % O₂, 3 % CO₂) of 1-MCP treated and control 'Jonagold' apple (Experiment 2). The shaded areas represents RA storage at 1 °C before CA is introduced. The error bars denote the standard error of the means (n=5).

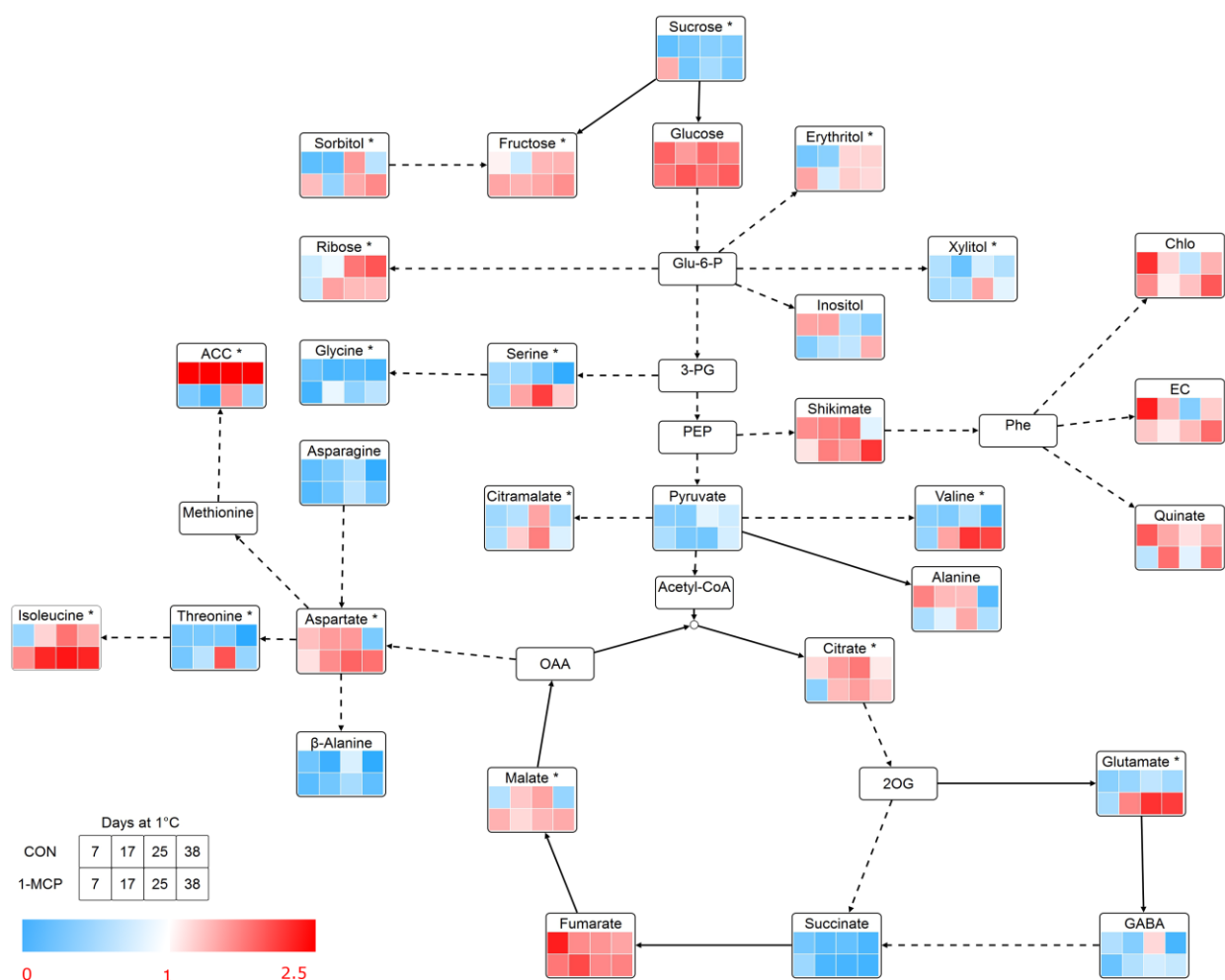


Fig. 7. Heat map showing the relative change of polar metabolites extracted from ‘Jonagold’ apples during five weeks of RA storage at 1°C (Experiment 2). The colour of the boxes represent the mean (n=5) of the relative change of each metabolite from their value at harvest. The upper boxes indicate the time profile of the control fruit while the lower boxes denote 1-MCP treated fruit. The red colour denotes the metabolites that are higher at that particular time point relative to harvest, and blue colour denotes the converse. Metabolites that showed significant difference between the two treatments in paired t-test are marked with asterisks. Abbreviations: Glu-6-P, Glucose-6-phosphate; 3-PG, 3-Phosphoglycerate; PEP, Phosphoenolpyruvate; Acetyl-CoA, Acetyl coenzyme A; 2OG, 2-oxolglutarate; OAA, Oxaloacetic acid; Phe, Phenylalanine and others as given in Table 1.

